Impact of *Bactrocera oleae* on the fungal microbiota of ripe olive drupes

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Abstract

The olive fruit fly (OFF), *Bactrocera oleae* is the most devastating pest affecting olive fruit worldwide. Previous investigations have addressed the fungal microbiome associated with olive drupes or *B. oleae*, but the impact of the insect on fungal communities of olive fruit remains undescribed. In the present work, the fungal microbiome of olive drupes, infested and non-infested by the OFF, was investigated in four different localities and cultivars. Olive fruit fly infestations caused a general reduction of the fungal diversity, a higher quantity of the total DNA and an increase in taxa that remained unidentified or had unknown roles. The infestations led to imbalanced fungal communities with the growth of taxa that are usually outcompeted. While it was difficult to establish a cause-effect link between fly infestation and specific fungi, it is clear that the fly alters the natural microbial balance, especially the low abundant taxa. On the other hand, the most abundant ones, were not significantly influenced by the insect. In fact, despite the slight variation between the sampling locations, *Aureobasidium*, *Cladosporium*, and *Alternaria*, were the dominant genera, suggesting the existence of a typical olive fungal microbiome.

Introduction

Olive (*Olea europaea* L.) is one of the most important cultivated crops on a global scale [1]. The countries of the coastal areas of the Mediterranean Basin represent the typical olive zone with more than 10 million ha, accounting for about 80% of the world's total olive cultivation area [2]. This crop is threatened by several abiotic and biotic stresses mainly caused by insects, pathogens, and nematodes [3–5]. Among those, the olive fruit fly (OFF) *Bactrocera oleae* (Rossi) is considered the most destructive key pest for olive production, and can be virtually found wherever *Olea* species are present [6]. *Bactrocera oleae*, in contrast to other Tephritidae Diptera, is strictly monophagous, feeding exclusively on olive fruits, and has a unique ability to feed on unripe green fruits which contain high levels of phenolic compounds such as oleuropein. This ability is attributed to the high number of detoxifying genes activated during larvae

feeding [7] as well as the presence of the symbionts such as 'Candidatus Erwinia dacicola' in their gut [8]. The microbiome of *B. oleae* has been found to be essential for its survival in natural conditions and is likely to play an important role on the longevity, competitiveness, and ability of the insect to cope with biotic stresses [9]. Although the source of *B. oleae* microbiome is unknown, some evidence suggests a vertical transmission from one generation to the next. Adult females of *B. oleae*, use their ovipositors to lay eggs inside olive drupes, and the hatched larvae form tunnels inside the fruit while feeding on the mesocarp. The physical damage, represented in fruit punctures and larvae feedings affect both crop yield and fruit and oil quality dramatically. In particular, *B. oleae* infestations affect phenolic content, fatty acids profile, and peroxide values of olive oil [10]. As a response to *B. oleae* infestation, the plant produces different defensive compounds including phytohormones, volatile organic compounds, and defense proteins, among others [11]. It has been also reported that the insect can facilitate secondary microbial infections caused by different microorganisms [10]. Despite the fact that these secondary colorizers are likely to affect olive oil quality, little is currently known about the impact of olive fly infestations on olive microbial communities. The role of fungi is particularly relevant in this context due to their importance as plant pathogens[12], and capability to produce mycotoxins. Indeed, recent microbiome studies have mainly focused on bacterial and, to a lesser extent, fungal communities associated with *B. oleae* [13]. Other investigations have focused on the fungal communities associated with different organs of the olive canopy including ripe drupes [14] but the impact of *B. oleae* on fungal communities has never been investigated. In the present study, the relationship between insect infestation and fungi has been analyzed by determining fungal diversity and total fungal DNA in infested and non-infested drupes from different cultivation areas in Italy.

Materials and methods

Ethics statement

No specific permissions were required for field activities since they only consisted in the collection of a small quantity of olive drupes having an irrelevant economic value. This work did not involve endangered or protected species.

Sampling and DNA extraction from olive drupes

Samples, were collected in the middle of November 2015 from four commercial olive orchards with some of the most important cultivars grown in Southern Italy: Leccino, Ottobratica, Carolea, and Ciciarello. Orchards were located in Monopoli (Puglia, Southern Italy), and in Mileto, Filadelfia and Maierato (Calabria, Southern Italy), respectively (Table 1).

From each location, 5 biological replicates of both infested and non-infested olives were randomly collected from an area of approximately 1 ha. Each biological replicate consisted of ten drupes collected from 5 different trees. Collected olives were quickly returned to the laboratory in plastic containers and stored at 4°C for no longer than 24 h prior to lyophilization (Labconco FreeZone 2.5). Before lyophilization the fruits' mesocarp and exocarp were

Table 1. Source of infested and non-infested olive samples analyzed in the present study.

Locality	Cultivar	Age (years)	GPS coordinates
Monopoli, Bari	Leccino	20	40°53'09.5"N 17°18'22.6"E
Mileto, Vibo Valentia	Ottobratica	30	38°36'05.4"N 16°04'06.4"E
Filadelfia	Carolea	30	38°47'33.9"N 16°16'55.1"E
Maierato	Ciciarello	30	38°43'17.5"N 16°12'42.6"E

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separated from the stone, which was discarded. Lyophilized samples were grounded in liquid nitrogen with sterile mortars and pestles, and total DNA was extracted from 80 mg of homogenate tissues as described by Mosca and co-workers [15]. Concentration and quality of extracted DNA were assessed using Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., USA). All samples were diluted to have a DNA concentration of 20 μg·μl^{-1}.

Quantification of total fungal DNA

Total fungal DNA was estimated by quantitative real-time PCR using the universal fungal primers ITS3_KYO2-ITS4 targeting the fungal ITS2 region of the rDNA [16] and the SYBR chemistry (Applied Biosystems, USA). To construct a representative standard curve, total DNA was extracted from a pure culture of *Colletotrichum acutatum sensu stricto* as described by Schena & Cooke [17], quantified using a Nanodrop spectrophotometer, and serially diluted with sterile water to yield final DNA concentrations ranging from 10 ng/μl to 100 fg/μl. PCR reactions were performed in a total volume of 20 μl containing 10 μl SYBR Green PCR Master Mix, 300 nM of each primer, and 2 μl of DNA template (20 ng/μl). All amplifications were performed in triplicate and water replaced template DNA in negative control reactions. Amplifications were performed and monitored using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) and data acquisition, and analysis was made using the supplied software according to the manufacturer's instructions. A standard curve was generated by plotting the DNA quantities against the corresponding Cq value. Correlation coefficients, mathematical functions, and PCR efficiencies were calculated and visualized using StepOne software.

The total quantity of fungal DNA in olive samples was quantified by amplifying 2 μl of DNA extracted from each biological replicate. The Cq value obtained for each biological replicate was an average of the three technical replicates. To take into account small variations in sample size and efficiency of extraction and amplification, the data were normalized using a qPCR method based on two universal primers and a probe [17]. Specifically, for each biological replicate, Cq values obtained with fungal universal primers were corrected against Cq values of the universal method (Cq-u) by multiplying the uncorrected Cq values by the ratio of the average of Cq-u and the Cq-u of each specific sub-sample. The concentration of total fungal DNA in unknown samples was extrapolated using corrected Cq values and the specific mathematical functions (regression solutions) determined for the standard curves. The total DNA concentration was expressed as ng of fungal DNA per g of fresh olive tissue.

Data were submitted to statistical analysis using the total DNA quantity as dependent variable and the location of sampling or the OFF infestations as fixed factors using SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). In the first case, analyses were separately performed for infested and non-infested olives and means were compared by a two-sided test pairwise comparison. In the latter case, analyses were performed for each locality by the ANOVA. Furthermore, a two-factor essay (general linear model) was performed in order to determine the effect of location and fly infestations and their interactions on the total fungal quantity.

Metabarcoding analyses

Library preparation and sequencing. DNA extracts were amplified using the universal fungal primers ITS3_KYO2 (GATGAAGAACGYAGYRAA) [16] and ITS4 (TCCTCCGCTTATT GATATGC) [18], modified by adding the adapters needed for Illumina MiSeq sequencing. PCR reactions were conducted in a total volume of 25 μl, consisting of 1 μl of DNA (50 μg) or nuclease-free water (QIAGEN, Valencia, CA, USA) as negative control, 12.5 μl of KAPA HiFi

HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) and 1.5 µl of each primer (10 µM). Amplification was performed in an Eppendorf Vapo Protect Mastercycler pro S (Eppendorf, Germany) as follows: 3 min at 98˚C followed by 30 cycles of 30 s at 95˚C, 30 s at 50˚C and 30 s at 72˚C. PCR products were purified using the Agencourt Ampure XP kit (BECKMAN COULTER-Beverly, Massachusetts), multiplexed using the Nextera XT indexing kit (Illumina, Inc., San Diego, CA), and purified again with the Agencourt Ampure XP kit. All purification and amplification reactions were performed according to the manufacturer's instructions. The concentration of purified amplicons was measured using Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts) and normalized to a common concentration of 20 ng/µl. Normalized amplicons were pooled by adding 10 µl of each product and kept at -20˚C until sequencing. Sequencing was performed on an Illumina MiSeq platform by loading 4 nM of denatured DNA into V3 MiSeq flow cell for 600 cycles (300 x 2) according to Illumina standard protocol.

Analysis of metabarcoding data

Raw Illumina data were processed using the Trimmomatic V0.32 [19] and PANDAseq assembler V2.11 [20] to eliminate low quality reads and merge paired-end sequences with a minimum of 100 bp read length. Chimeric sequences were identified and removed using VSEARCH 1.4.0 [21]. The downstream analyses were carried out using QIIME 1.9.1 pipeline [22] as described by Abdelfattah and co-workers [23]. The operational taxonomic Units (OTUs) table was normalized by rarefaction to an even sequencing depth in order to remove sample heterogeneity. The rarefied OTU table was used to calculate alpha diversity indices including Observed Species (*Sobs*) and Shannon metrics and to plot taxa relative abundances (RA). MetagenomeSeq's cumulative sum scaling (CSS) [24] was used as a normalization method for other downstream analyses. Alpha diversities were compared based on a two-sample t-test using non-parametric (Monte Carlo) methods and 999 Monte Carlo permutations (999). The CSS normalized OTU table was used to calculate the β-diversity using the Bray Curtis metrics [25] and construct the principal coordinate analysis (PCoA) plots [26]. Beta diversity comparisons were done using The ANalysis Of SIMilarity (ANOSIM) method [27]. To evaluate the significance of differences in RAs of detected taxa, the most abundant ones ($\geq$ 0.1%) were compared using the Kruskal-Wallis test [28]. In all tests, significance was determined using 1000 Monte Carlo permutations, and the Benjamini-Hochberg (FDR) corrections were used to adjust the calculated p-values.

Results

Construction of a standard curve for quantitative analyses

Total fungal DNA was estimated by quantitative real-time PCR using the universal fungal primers. Standard curve generated using serial dilutions of DNA of *C. acutatum* revealed a linear correlation over six orders of magnitude (from 10 ng to 100 fg) with a correlation coefficient (R^2) of 0.997. A specific linear equation (y = 20.31–3.30x) was established and utilized to quantify the total fungal DNA in olive drupes.

Fungal diversity and richness

After quality filtering, Illumina high throughput sequencing resulted in a total of 3,019,642 reads, with a mean of 86,275 reads per sample. Overall, these sequences were assigned to 1,428 OTUs (97% similarity threshold) with a mean of 123 OTUs per biological replicate. According to the taxonomy assignments, the OTUs were assigned to the phyla *Ascomycota* (89.9%),

Fig 1. Sunburst chart showing the total relative abundance of fungal phyla (interior circle) and classes (exterior circle) overall detected in infested and non-infested olives in the four investigated localities.

Basidiomycota (9.7%), and a very small fraction to *Zygomycota* (0.01%) and unidentified fungi (0.3%) (Fig 1).

Most *Ascomycota* were represented in the classes *Dothideomycetes* (70.5%) and *Sordariomycetes* (8.4%), whereas the majority of the *Basidiomycota* was represented by an unidentified

class (2.9%), *Agaricomycetes* (2.5%), and *Tremellomycetes* (2.1%) (Fig 1). At the genus level, the most abundant genera were *Aureobasidium* (30.6%), *Cladosporium* (17.2%), *Alternaria* (11.8%), *Colletotrichum* (4.5%), Unidentified Ascomycota (3.7%), and *Pseudocercospora* (3.6%) (Figs 2 and 5).

Comparison of locations

Quantitative real-time PCR analyses of olive drupes from four commercial orchards located in Monopoli, Mileto, Filadelfia and Maierato, revealed significant differences in the total quantity of fungal DNA according to the investigated locations (Table 1 and Fig 3).

However, differences were more remarkable in infested olives as compared to the non-infested ones. In the first case, significant differences were revealed between most samples since the total quantity of detected DNA per g of fresh tissue ranged between 21400 (Monopoli) and 717 ng (Filadelfia). In the latter case, most samples were not significantly different and total DNA ranged from 2787 ng and 341 ng (Fig 3). According to metabarcoding analyses, a consistent fungal community was associated with olive drupes in all investigated localities since three genera (*Aureobasidium*, *Cladosporium* and *Alternaria*) cumulatively accounting for approximately 60% of the detected sequences, were always the prevalent fungi (Fig 2).

On the other hand, the number of observed species (*sobs* index) differed significantly in the four sampling locations, with the exception of Maierato and Filadelfia (Table 2). A lower level of discrimination was provided by the Shannon index, since significant differences were only revealed between Mileto and both Filadelfia and Monopoli (Table 2).
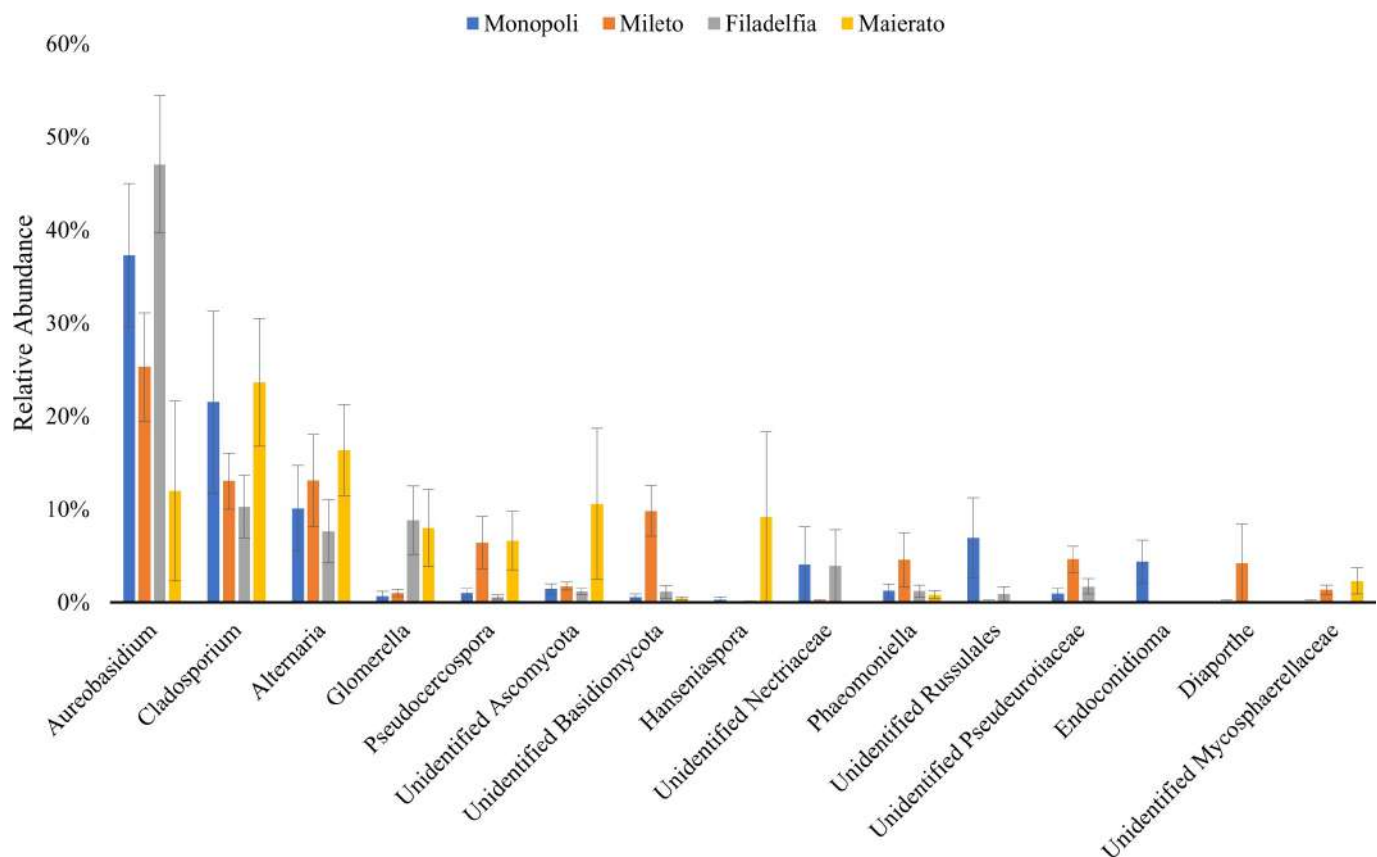

Fig 2. Relative abundances of the dominant fungal genera (RA ≥1%) detected in infested and non-infested olives in Monopoli, Mileto, Filadelfia, and Maierato. Values are average of the taxa relative abundance ± the standard error.
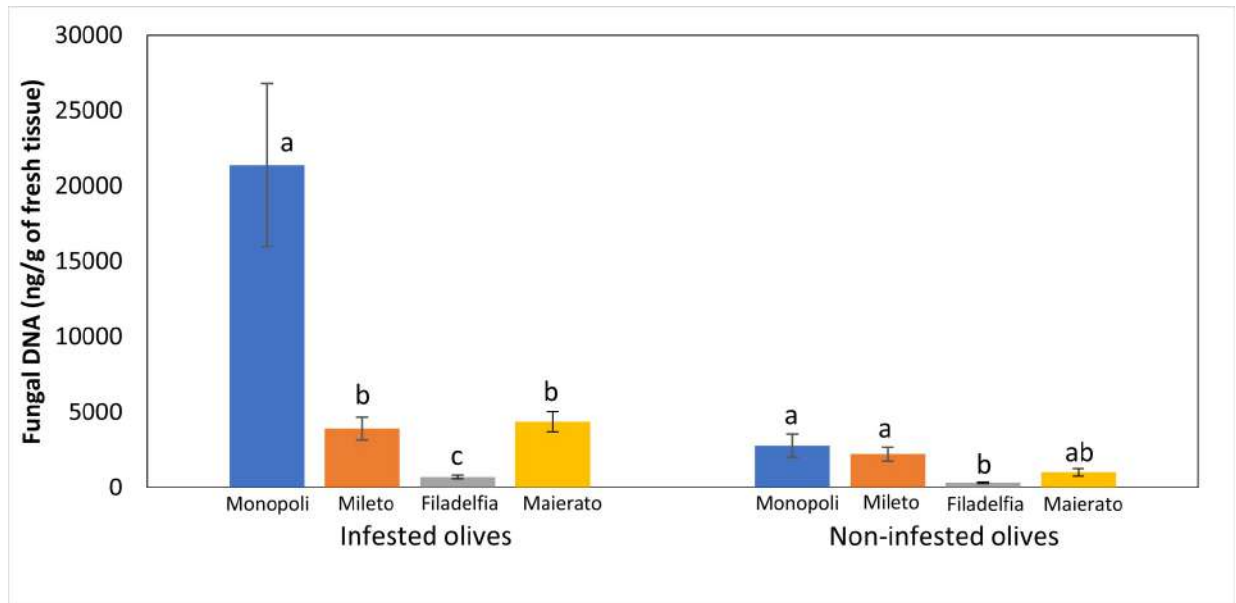
Fig 3. Estimated concentration of fungal DNA in olive samples collected in Monopoli, Mileto, Filadelfia and Maierato. Independent statistical analyses were performed for infested (left side) and non-infested (right side) olive samples. Different letters indicate significantly different values according to 2-sided tests ($P \leq 0.05$). Bars indicate standard errors of the means.

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In particular, significant differences among investigated localities were revealed for 21 taxonomic entities that comprised both abundantly detected taxa such as *Aureobasidium*, *Colletotrichum* and *Pseudocercospora* and much less abundant taxa (Table 3).

Furthermore, the analysis of beta diversity showed that the fungal community significantly differed between all the sampling locations (Table 4).

These differences were particularly evident when beta diversity results were visualized on a PCoA plot since samples from all localities segregated separately (Fig 4).

Comparison of infested and non-infested olives

The total quantity of fungal DNA was higher in infested olives as compared to the non-infested ones in all localities. In particular, total fungal DNA increased by 87.0, 43.6, 52.4, and 76.5%, in Monopoli, Mileto, Filadelfia, and Maierato, respectively, although differences were statistically significant only in Monopoli and Maierato (Fig 4).

The ANOVA two-factor essay confirmed that the fungal DNA quantity varied depending on the fly puncture ($F = 18,541$; df = 1; $P<0.001$), the site ($F = 13,474$; df = 3; $P < 0.001$), and the interaction of both factors ($F = 9,254$; df = 3; $P<0.001$).

Table 2. Comparison of the alpha diversity of the fungal communities associated to olive drupes in the four investigated localities (Monopoli, Mileto, Filadelfia, and Maierato) according to Shannon and observed species (*sobs*) indexes. P values of the pair-wise comparisons were determined using student-t-test and 999 Monte Carlo permutations. Both Shannon and Sobs Mean values are reported in bold for each investigated locality.

	Shannon				sobs			
	Monopoli	**Mileto**	**Filadelfia**	**Maierato**	**Monopoli**	**Mileto**	**Filadelfia**	**Maierato**
Monopoli	**2.08**	0.04	0.47	0.68	**28.56**	0.015	0.024	0.006
Mileto		**3.23**	0.03	0.05		**63.90**	0.024	0.045
Filadelfia			**2.34**	0.81			**36.91**	0.115
Maierato				**2.27**				**42.74**

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Table 3. List of fungal taxa characterized by a significantly different relative abundance (RA) between the four investigated locations. P values were calculated using nonparametric test of Kruskal-Wallis through 1000 permutations. FDR P are the P value corrected by the Benjamini-Hochberg FDR procedure.

Detected taxa	FDR P	Relative abundance (%)			
		Monopoli	Mileto	Filadelfia	Maierato
Aureobasidium	0.01232	39.11	26.66	48.91	12.18
Colletotrichum	0.00887	0.18	1.05	9.14	8.36
Pseudocercospora	0.04381	0.69	6.88	0.61	6.91
Unidentified *Basidiomycota*	0.00621	0.27	10.80	1.18	0.45
Unidentified *Pseudeurotiaceae*	0.00156	0.86	5.12	1.80	0.00
Endoconidioma	0.00029	4.93	0.04	0.03	0.00
Diaporthe	0.00533	0.00	4.24	0.03	0.00
Unidentified *Mycosphaerellaceae*	0.00156	0.00	1.42	0.00	2.44
Quambalaria	0.00621	0.10	0.00	2.96	0.00
Fusicladium	0.00029	0.00	0.01	0.91	2.05
Sporobolomyces	0.00666	1.38	0.56	0.80	0.00
Botryosphaeria	0.00404	0.00	2.13	0.00	0.00
Dioszegia	0.00263	0.00	0.01	1.87	0.00
Stemphylium	0.00130	0.09	0.21	0.00	1.44
Plenodomus	0.01232	0.00	0.00	1.52	0.00
Unidentified Fungi	0.02754	1.01	0.03	0.25	0.00
Taphrina	0.00072	0.00	1.01	0.00	0.06
Unidentified *Tremellales*	0.00079	0.00	0.75	0.00	0.00
Unidentified *Tremellomycetes*	0.00621	0.56	0.00	0.00	0.00
Tilletiopsis	0.03415	0.00	0.01	0.00	0.46
Toxicocladosporium	0.00031	0.00	0.00	0.00	0.43

A significant impact of the OFF on the composition of the fungal community was confirmed by metagenomic analyses. Regardless of the location, the number of observed species was significantly higher in non-infested fruits with a mean of 49.1 OTUs compared to 35.7 OTUs in infested fruits (P = 0.025). Similarly, healthy fruits had a higher Shannon index value (2.75) compared to infested fruits (2.2) (P = 0.020). Despite these differences, both the number of observed species and Shannon index were not statistically significant when infested and non-infested fruits were compared individually (Table 4). Interestingly, most of the taxa with significant different RA in infested and non-infested olives had a quite low RA and/or remained unidentified (Table 5). On the other hand, the most abundant taxa such as *Aureobasidium*, *Cladosporium* and *Alternaria*, did not differ significantly in their RAs between infested and non-infested olives (Table 5).

Table 4. Comparison of alpha and beta diversity in infested and non-infested olive drupes collected in Monopoli, Mileto, Filadelfia, or Maierato, according to Shannon and observed species (*sobs*) indexes. Significance was determined according to the nonparametric test ANOSIM for beta diversity and student t-test for Shannon and observed species (*sobs*). P-values were determined using 999 Monte Carlo permutations.

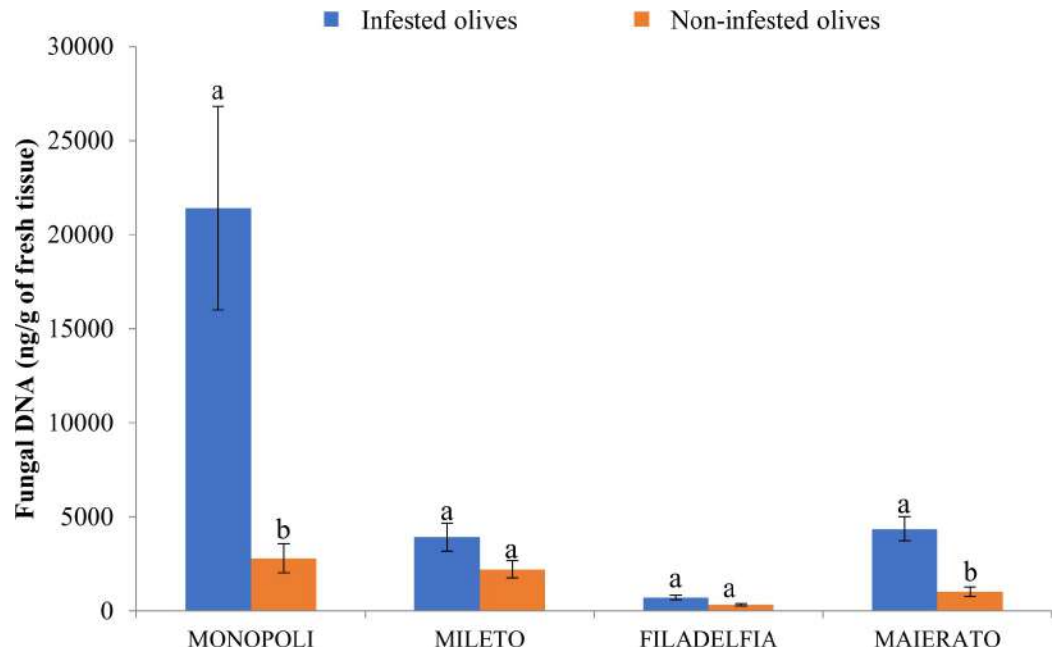|	Observed species (*sobs*)		Shannon		P value		
	Infected	Non-infested	Infected	Non-infested	Sobs	Shannon	Beta diversity
Monopoli	25.8	31.3	2.11	2.05	0.18	0.95	0.033
Mileto	38.2	84.5	2.50	3.82	0.07	0.06	0.005
Filadelfia	39.7	33.4	2.30	2.38	0.25	0.90	0.016
Maierato	39.0	47.4	1.91	2.73	0.19	0.27	0.029
All locations	36.1	51.2	2.19	2.80	0.025	0.02	0.001

Fig 4. Comparison of total fungal DNA in infested and non-infested olive drupes in the four investigated localities (Monopoli, Mileto, Filadelfia and Maierato). Independent statistical analyses were performed for each locality. Different letters indicate significantly different values according to ANOVA univariate analysis of variance (P ≤ 0.05). Bars indicate standard errors of the means.

In particular, most abundant taxa showed different and inconsistent trends in infested and non-infested olives according to the sampling localization (Fig 5). For instance, the genus *Aureobasidium* had a higher RA in infested olives in Filadelfia, Mileto and Maierato and lower abundance in Monopoli. The genus *Cladosporium* was more abundant in infested olives in Filadelfia and Mileto and less abundant in Monopoli and Maierato. The genus *Alternaria* was more abundant in infested olives in Monopoli and more abundant in non-infested drupes in the other sampling sites (Fig 5).

A clearer picture of the impact of the OFF on olive fungal community was achieved through the beta diversity analysis. In fact, *B. oleae* infestations caused a significant change in the fungal community structure in all investigated locations (Filadelfia P = 0.016, Maierato P = 0.029, Mileto P = 0.005, and Monopoli P = 0.033) and differences were confirmed by cumulatively analyzing drupes from the four sampling sites (P = 0.001) (Table 4). In agreement with the beta diversity analyses, the PCoA visualization of results showed a separate clustering of infested and non-infested samples in all the investigated localities (Fig 6).

Discussion

The present work enabled the detailed investigation of the impact of *B. oleae* on the structure of the fungal community of the olive carposphere. Interestingly, OFF infestations resulted in a general reduction in fungal diversity, but also in a significant increase in the total quantity of fungi. Although these results were shared by all investigated localities, the impact of OFF on the fungal community structure varied in the investigated localities. It appeared that flies had a unique influence in each locality in relation to different environmental conditions, cultivars, and management practices. A significant impact of *B. oleae* infestation on the structure of fungal communities was confirmed by beta diversity analyses since they showed a clear separation

Detected taxa	Monopoli			Mileto			Filadelfia			Maierato			Average
	Fly -	Fly +	Trend	Fly -	Fly +	Trend	Fly -	Fly +	Trend	Fly -	Fly +	Trend	
Aureobasidium	46.6	29.7	↓	16.1	36.8	↑	42	51.1	↑	4	18.3	↑	30.58
Cladosporium	25.2	18.9	↓	12.7	13.5	↑	2.4	16.6	↑	31.4	17.4	↓	17.26
Alternaria	2.7	17.1	↑	15.1	10.5	↓	11.8	4.3	↓	26	8.6	↓	12.01
Colletotrichum	-	-	-	1.8	-	↓	16.5	2.7	↓	9.4	6.9	↓	4.66
Pseudocercospora	1.2	-	↓	6.9	5.8	↓	0.7	0.5	↓	5.4	7.7	↑	3.53
Unidentified Ascomycota	1.5	0.9	↓	2.3	1	↓	1	1.3	↑	4.6	15.4	↑	3.5
Unidentified Basidiomycota	-	-	-	16.2	1.9	↓	-	2	↑	0.6	-	↓	2.59
Unidentified Russulales	-	15.1	↑	-	1.3	↑	-	1.6	↑	-	-	-	2.25
Unidentified Nectriaceae	-	8.6	↑	-	-	-	8.8	-	↓	-	-	-	2.18
Hanseniaspora	-	-	-	-	-	-	-	-	-	-	16.5	↑	2.06
Phaeomoniella	-	2.2	↑	0.6	9.6	↑	-	2.1	↑	1.6	-	↓	2.01
Unidentified Pseudeurotiaceae	1.7	-	↓	7.8	0.7	↓	2.6	1.1	↓	-	-	-	1.74
Endoconidioma	8.3	1.3	↓	-	-	-	-	-	-	-	-	-	1.2
Diaporthe	-	-	-	-	9.4	↑	-	-	-	-	-	-	1.18
Unidentified Mycosphaerellaceae	-	-	-	2.4	-	↓	-	-	-	4.9	-	↓	0.91
Sporobolomyces	3.1	-	↓	1.8	-	↓	1.4	0.9	↓	-	-	-	0.9
Fusicladium	-	-	-	-	-	-	1.9	-	↓	3.6	0.6	↓	0.76
Quambalaria	-	-	-	-	-	-	0.7	4.5	↑	-	-	-	0.65
Botryosphaeria	-	-	-	-	4.7	↑	-	-	-	-	-	-	0.59
Didymella	-	-	-	-	-	-	-	4.4	↑	-	-	-	0.55
Cryptococcus	1.2	0.6	↓	0.7	1.3	↑	-	-	-	-	-	-	0.48
Dioszegia	-	-	-	-	-	-	3.7	-	↓	-	-	-	0.46
Phaeoramularia	-	-	-	2.2	-	↓	-	-	-	1	-	↓	0.4
Stemphylium	-	-	-	-	-	-	-	-	-	1.1	1.8	↑	0.36
Plenodomus	-	-	-	-	-	-	-	2.6	↑	-	-	-	0.33
Unidentified fungi	2.1	-	↓	-	-	-	-	-	-	-	-	-	0.26
Kwoniella	-	-	-	-	-	-	-	-	-	-	1.8	↑	0.23
Taphrina	-	-	-	1.7	-	↓	-	-	-	-	-	-	0.21
Mycoarthris	-	1.6	↑	-	-	-	-	-	-	-	-	-	0.2
Unidentified Dothideomycetes	-	-	-	-	-	-	1.5	-	↓	-	-	-	0.19
Penicillium	1.4	-		-	-	-	-	-	-	-	-	-	0.18
Eutypa	-	1.3	↑	-	-	-	-	-	-	-	-	-	0.16

Fig 5. Heatmap highlighting the differences in the taxa detected in infested (Fly +) and non-infested (Fly -) olives from four different localities with an overall relative abundance of at least 0.16%. Arrows indicate the trend of each taxa in infested olives as compared to the non-infested ones.

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of infested and non-infested samples in all investigated locations. Interestingly, differences were more noticeable in taxa with a quite low RA while abundant taxa such as *Aureobasidium*, *Cladosporium*, and *Alternaria*, did not differ significantly and showed inconsistent trends in

Table 5. List of taxa with a significant different relative abundance (RA) in infected and non-infected olive drupes, in the four investigated locations.

Location	Taxa	P-value	Relative abundance (%)	
			Non-infested	Infested
Monopoli	Unidentified *Tremellomycetes*	0.0139	1.12	0.00
	Unidentified *Pseudeurotiaceae*	0.0180	1.72	0.01
	Unidentified *Russulales*	0.0180	0.01	15.20
	Quambalaria	0.0180	0.01	0.20
	Unidentified *Basidiomycota*	0.0180	0.53	0.01
	Unidentified Fungi	0.0180	2.01	0.01
	Unidentified *Sporobolomyces*	0.0202	2.74	0.02
Mileto	*Sporobolomyces*	0.0105	1.01	0.00
	Phaeoramularia	0.0127	2.46	0.08
	Colletotrichum	0.0127	1.88	0.01
	Unidentified *Mycosphaerellaceae*	0.0143	2.53	0.04
	Unidentified *Pseudeurotiaceae*	0.0143	8.64	0.72
	Taphrina	0.0143	1.76	0.06
	Unidentified *Basidiomycota*	0.0143	17.81	2.04
	Unidentified *Nectriaceae*	0.0318	0.32	0.00
	Phaeomoniella	0.0491	0.65	10.11
Filadelfia	Unidentified *Dothideomycetes*	0.0127	1.56	0.03
	Cryptococcus	0.0139	0.03	0.45
	Unidentified *Basidiomycota*	0.0139	0.08	2.06
	Plenodomus	0.0318	0.00	2.74
Maierato	*Toxicocladosporium*	0.0139	0.04	0.92
	Fusicladium	0.0275	0.62	3.84

the different sampling locations. Overall, the obtained data suggest that OFF infestations led to imbalanced fungal communities. Indeed, while it is difficult to make concrete statements on the role of specific fungal genera in infested fruits, the reduced microbial diversity and the higher quantity of fungal DNA support the hypothesis of an altered equilibrium among taxa. The abundant presence of taxa with unknown roles as well as unidentified taxa indicate that the infestation of OFF may have facilitated the colonization of the fruit by alien species and/or favored the growth of taxa that are usually outcompeted. Interestingly, consistent results were obtained in four different localities characterized by different environmental conditions, cultivars and management practices, supporting a similar impact of OFF in altering fungal communities. While according to results of the present study it is difficult to establish a cause-effect link between fly and specific fungal taxa, it is clear that the fly alters the natural microbial balance and this may be linked to the reduced oil quality made from infested fruits [29].

The consensus of the olive community composition described herein, including the proportion of the fungal taxa at all the taxonomical levels (from phyla to genera) is in strong agreement with the previous description of the olive fungal community; being dominated by fungal genera such as *Aureobasidium*, *Cladosporium*, and *Alternaria* [14]. The stability of the olive fungal community in the investigated samples in the present and in a previous study [14], and how they differ remarkably from other hosts [23,30], confirms that the microbial community is determined primarily by its host identity. Interestingly, non-pathogenic fungal species such as *Aureobasidium pullulans* were largely prevalent in all localities. The abundant presence of this species is remarkable since this yeast-like fungus is well-known for its antimicrobial activity against a wide range of microbes [31–33] and has been used as biological control agent
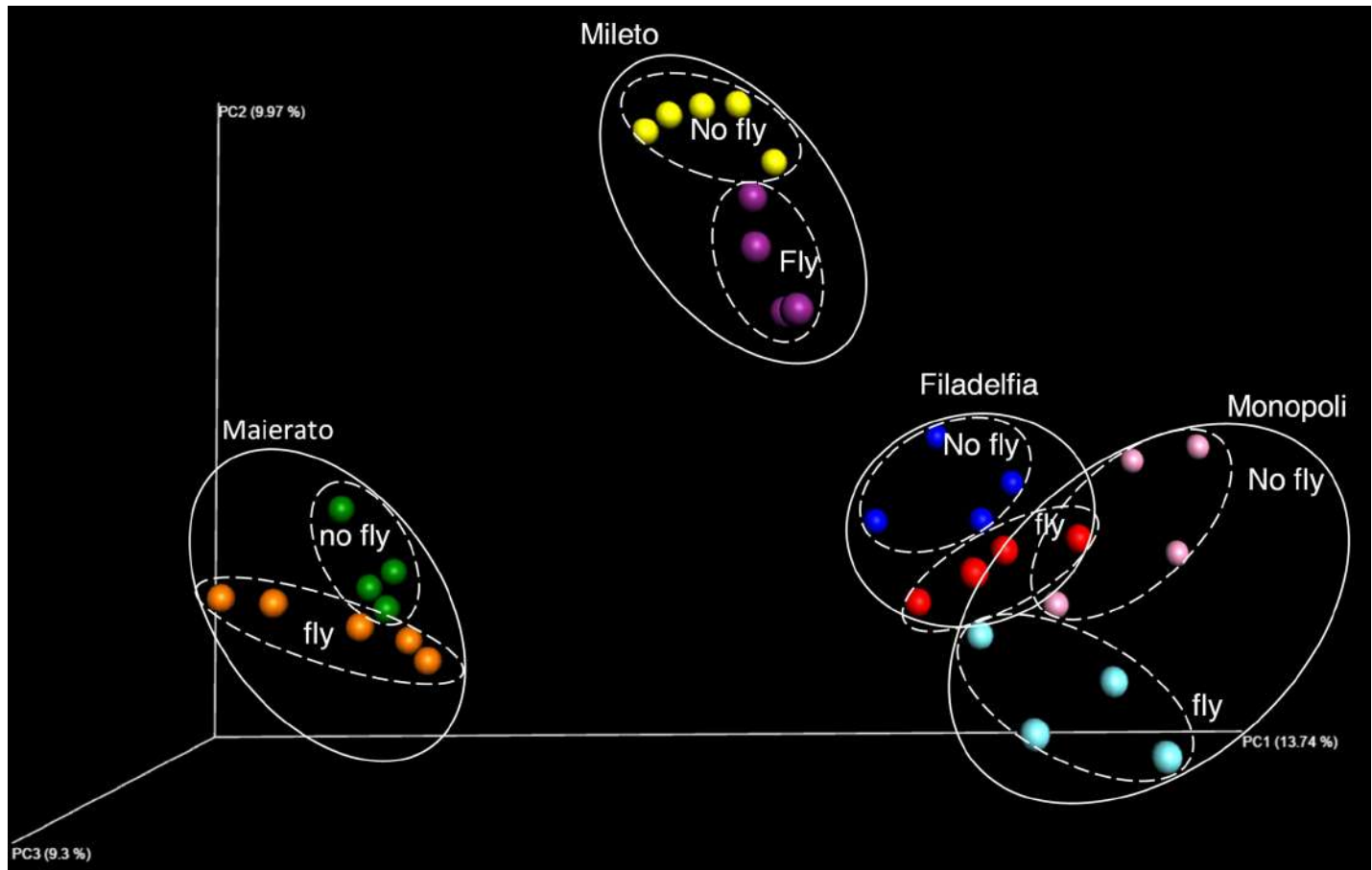
Fig 6. Principal coordinate analysis (PCoA) of the fungal communities associated with olive drupes infested (fly) or non-infested (no fly) by the olive fruit fly Bactrocera oleae and collected in the four studied localities (Monopli, Mileto, Filadelfia or Maierato).

against several plant diseases [34–36]. Regardless of the hypothetical role of *Aureobasidium*, little is known about the actual ecological reasons for its natural prevalence on olive plants as compared to other plant species.

Despite the characteristic fungal community associated with olives, in the present study it was difficult to determine a precise list of fungal taxa that defines infested and non-infested olives, also because some significant variations were observed between the investigated locations/cultivars. It is likely that these differences were the results of several factors including environmental conditions, cultivars, and management practices. In fact, previous studies have showed that the geographical location, among other factors such as the management practices, can influence the fungal community of the host [23,37]. Indeed, it has been proven that different organs, and even different parts of the same organ can significantly impact the microbial composition [23].

Interestingly, the majority of olive fungal community, described herein and in the previous study [14] overlap with those found on olive fruit fly [13]. This is the first observation to document two different species, especially from two different kingdoms, to have a very similar fungal microbial community. In agreement with our results, the presence of several bacterial species common to both *B. oleae* and olive trees have already been reported [38]. Due to the limited information currently available on this subject, it is difficult to make concrete

speculations about the significance of our observation. However, since *B. oleae* spends a considerable part of its life cycle in association with olive tissue [10], the observed similarities may indicate a shared evolutionary process.

Supporting information

S1 Fig. Real-time PCR standard curve.
(PNG)

S1 Dataset. Fungal OTUs table.
(BIOM)

S2 Dataset. Normalized OTUs table by Cumulative Sum Scaling (CSS).
(BIOM)

S3 Dataset. Rarified OTUs table at even depth.
(BIOM)

S4 Dataset. Bray Curtis distance matrix.
(TXT)

S5 Dataset. Principle coordinates.
(TXT)

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References

1. Loumou A, Giourga C (2003) Olive groves: "The life and identity of the Mediterranean". Agric Human Values 20: 87–95.

2. FAOSTAT (2017) Food and Agriculture Organization of the United Nations. Food and Agriculture Organization of the United Nations.

3. López-Escudero FJ, Mercado-Blanco J (2011) Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344: 1–50.

4. Sanzani SM, Schena L, Nigro F, Sergeeva V, Ippolito A, et al. (2012) Abiotic diseases of olive. J Plant Pathol 94: 469–491.

5. Cacciola SO, Faedda R, Agosteo GE (2011) Olive anthracnose. In: Schena L, Agosteo GE, Cacciola SO, editors. Olive diseases and Disorders. India Transworld Research Network. pp. 223–246.

6. Gutierrez AP, Ponti L, Cossu QA (2009) Effects of climate warming on olive and olive fly (*Bactrocera oleae* (Gmelin)) in California and Italy. Clim Change 95: 195–217.

7. Pavlidi N, Dermauw W, Rombauts S, Chrisargiris A, Van Leeuwen T, et al. (2013) Analysis of the olive fruit fly *Bactrocera oleae* transcriptome and phylogenetic classification of the major detoxification gene families. PLoS One 8: e66533. https://doi.org/10.1371/journal.pone.0066533 PMID: 23824998

8. Pavlidi N, Gioti A, Wybouw N, Dermauw W, Ben-Yosef M, et al. (2017) Transcriptomic responses of the olive fruit fly *Bactrocera oleae* and its symbiont *Candidatus Erwinia dacicola* to olive feeding. Sci Rep 7: 42633. https://doi.org/10.1038/srep42633 PMID: 28225009

9. Ras E, Beukeboom LW, Cáceres C, Bourtzis K (2017) Review of the role of gut microbiota in mass rearing of the olive fruit fly, *Bactrocera oleae*, and its parasitoids. Entomol Exp Appl 164: 237–256.

10. Malheiro R, Casal S, Baptista P, Pereira JA (2015) A review of *Bactrocera oleae* (Rossi) impact in olive products: From the tree to the table. Trends Food Sci Technol 44: 226–242.

11. Alagna F, Kallenbach M, Pompa A, De Marchis F, Rao R, et al. (2016) Olive fruits infested with olive fly larvae respond with an ethylene burst and the emission of specific volatiles. J Integr Plant Biol 58: 413–425. https://doi.org/10.1111/jipb.12343 PMID: 25727685

12. Abdelfattah A, Malacrinò A, Wisniewski M, Cacciola SO, Schena L (2018) Metabarcoding: A powerful tool to investigate microbial communities and shape future plant protection strategies. Biol Control 120: 1–10.

13. Malacrinò A, Schena L, Campolo O, Laudani F, Mosca S, et al. (2017) A metabarcoding survey on the fungal microbiota associated to the olive fruit fly. Microb Ecol 73: 677–684. https://doi.org/10.1007/s00248-016-0864-z PMID: 27687872

14. Abdelfattah A, Nicosia MGLD, Cacciola SO, Droby S, Schena L (2015) Metabarcoding analysis of fungal diversity in the phyllosphere and carposphere of olive (*Olea europaea*). PLoS One 10: e0131069. https://doi.org/10.1371/journal.pone.0131069 PMID: 26132745

15. Mosca S, Li Destri Nicosia MG, Cacciola SO, Schena L (2014) Molecular analysis of *Colletotrichum* species in the carposphere and phyllosphere of olive. PLoS One 9: e114031. https://doi.org/10.1371/journal.pone.0114031 PMID: 25501572

16. Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLoS One 7: e40863. https://doi.org/10.1371/journal.pone.0040863 PMID: 22808280

17. Schena L, Cooke DEL (2006) Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. J Microbiol Methods 67: 70–85. https://doi.org/10.1016/j.mimet.2006.03.003 PMID: 16603267

18. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand D, Sninsky J, White T, editors. PCR protocols: a guide to methods and applications. New York: Academic Press, Inc. pp. 315–322.

19. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

20. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD (2012) PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 13: 31. https://doi.org/10.1186/1471-2105-13-31 PMID: 22333067

21. Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ Preprints 4: e2409v2401.

22. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335–336. https://doi.org/10.1038/nmeth.f.303 PMID: 20383131

23. Abdelfattah A, Wisniewski M, Droby S, Schena L (2016) Spatial and compositional variation in the fungal communities of organic and conventionally grown apple fruit at the consumer point-of-purchase. Hortic Res 3: 16047. https://doi.org/10.1038/hortres.2016.47 PMID: 27766161

24. Paulson JN, Stine OC, Bravo HC, Pop M (2013) Differential abundance analysis for microbial marker-gene surveys. Nat Methods 10: 1200–1202. https://doi.org/10.1038/nmeth.2658 PMID: 24076764

25. Bray JR, Curtis JT (1957) An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr 27: 325–349.

26. Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71: 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005 PMID: 16332807

27. Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18: 117–143.

28. Kruskal WH, Wallis WA (1952) Use of ranks in one-criterion variance analysis. J Am Stat Assoc 47: 583–621.

29. Torbati M, Arzanlou M, Azadmard-damirchi S, Babai-ahari A, Alijani S (2014) Effect of fungal species involved in the olive fruit rot on the qualitative properties of olive oil. Arch Phytopathol Plant Protect 47: 292–297.

30. Abdelfattah A, Cacciola SO, Mosca S, Zappia R, Schena L (2017) Analysis of the Fungal Diversity in Citrus Leaves with Greasy Spot Disease Symptoms. Microb Ecol 73: 739–749. https://doi.org/10.1007/s00248-016-0874-x PMID: 27752718

31. Chi Z, Wang F, Chi Z, Yue L, Liu G, et al. (2009) Bioproducts from Aureobasidium pullulans, a biotechnologically important yeast. Appl Microbiol Biotechnol 82: 793–804. https://doi.org/10.1007/s00253-009-1882-2 PMID: 19198830

32. Ippolito A, Schena L, Pentimone I, Nigro F (2005) Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. Postharvest Biol Technol 36: 245–252.

33. Wang FW, Jiao RH, Cheng AB, Tan SH, Song YC (2007) Antimicrobial potentials of endophytic fungi residing in *Quercus variabilis* and brefeldin A obtained from *Cladosporium* sp. World J Microbiol Biotechnol 23: 79–83.

34. Ippolito A, El Ghaouth A, Wilson CL, Wisniewski M (2000) Control of postharvest decay of apple fruit by *Aureobasidium pullulans* and induction of defense responses. Postharvest Biol Technol 19: 265–272.

35. Schena L, Nigro F, Pentimone I, Ligorio A, Ippolito A (2003) Control of postharvest rots of sweet cherries and table grapes with endophytic isolates of *Aureobasidium pullulans*. Postharvest Biol Technol 30: 209–220.

36. Pesce VM, Nally MC, Carrizo GP, Rojo C, Pérez BA, et al. (2017) Antifungal activity of native yeasts from different microenvironments against *Colletotrichum gloeosporioides* on ripe olive fruits. Biol Control 120: 43–51.

37. Finkel OM, Burch AY, Lindow SE, Post AF, Belkin S (2011) Geographical location determines the population structure in phyllosphere microbial communities of a salt-excreting desert tree. Appl Environ Microbiol 77: 7647–7655. https://doi.org/10.1128/AEM.05565-11 PMID: 21926212

38. Sacchetti P, Granchietti A, Landini S, Viti C, Giovannetti L, et al. (2008) Relationships between the olive fly and bacteria. J Appl Entomol 132: 682–689.